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EXPRESSION OF NRDR DIFFERENT ISOFORMS IN MICE UTERUS

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ABSTRACT

Uterus is an important reproductive organ for embryonic growth and development of viviparous animals. Its normal function is regulated by complex endocrine system and multiple genes. NRDR is a retinoic acid metabolizing enzyme with strong retinol oxidation and retinoaldehyde reduction activities. Its function in the reproductive system has been partially studied. In this study, the expression distribution and change of NRDR in in different physiological states and different developmental stages of mouse uterus will be clarified, so as to provide an experimental basis for further research on its function in uterus. Using immunohistochemistry and RT-qPCR, NRDR was proved mainly expressed in mouse endometrial by immunohistochemistry. The expression of two different isoforms NRDR-1 and NRDR-2 in mouse uterus increased gradually with the development of the mouse, and peaked at 15 days after birth. The expression of NRDR-1 has no significant difference during estrous and diestrus in mouse uterus. However, the expression of NRDR-2 in estrous period was significantly higher than that in diestrus; NRDR two isoforms increased during embryo recognition and implantation in mouse uterus, and their expression decreased after implantation. The experimental results of the above expression patterns indicated that NRDR played a very important role in the mouse uterus, which was related to the estrus cycle and embryo implantation, among which the isoforms NRDR-2 might play a major role. The above results provide a basis for further study of the function of NRDR in uterus. The detection of the expression of NRDR in the uterus can provide experimental basis for understanding the molecular mechanism of development and other physiological processes in uterus.

Keywords: NRDR; isoforms; uterus; mice

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INTRODUCTION

Uterus is an important reproductive organ of female animals. The normal development of the embryo depends on the microenvironment provided by the uterus and is regulated by the expression of various hormones and genes (Wang *et al.*, 2005). During embryo implantation, the uterus interacts with the embryo, and proliferation and differentiation of endometrial cells is necessary to maintain pregnancy (Fouladi Nashta *et al.*, 2004). The analysis of the expression variation of genes in uterus can provide a basis for the understanding of the pregnancy process and the mechanism of embryonic development.

NADP (H)-dependent retinol dehydrogenase/ reductase (NRDR) is encoded by the gene of short chain dehydrogenase family member 4 (DHRS4) gene, a member of the SDR superfamily. NRDR had high homology in humans, mice, pigs and other mammals (Du *et al.*, 2004). NRDR was first purified in rabbit liver 1997, and was also expressed in liver of other mammals (Sierra-Torres *et al.*, 2006). Previous studies found that NRDR had strong activities of retinol oxidation and retinal reduction (Lei *et al.*, 2003), and reduced other ketoaldehydes (Du *et al.*, 2004; Duester *et al.*, 1999). NRDR also acted as an enzyme that regulated the production and metabolism of retinoic acid in the body (Endo *et al.*, 2009; Kisiela *et al.*, 2011).

The expression of NRDR was regulated by non-coding RNA and DNA methylation in human (Lenschow *et al.*, 2007; Liang *et al.*, 2020; Su *et al.*, 2016; L. Wang *et al.*, 2021; Wardlaw *et al.*, 2022; Zhang *et al.*, 2009). Human DHRS4 gene had different splicing bodies, and different splicing bodies have different subcellular localization (Henkes *et al.*, 2015). Other research groups found that NRDR protein had strong retinol oxidation and retinol reduction activities and was a catalytic enzyme involved in retinoic acid synthesis and metabolism in vivo (Nguyen *et al.*, 2023; Wu *et al.*, 2023). NRDR had been proved to have the function of carbonyl catalytic reduction in different tissues (Afsar *et al.*, 2023; Alzahrani *et al.*, 2023). On the other hand, NRDR was related to the occurrence of multiple cancers (Amai *et al.*,

2020; Dai *et al.*, 2020; Song *et al.*, 2021). However, there are few studies on its function in the reproductive system. Previous studies have shown that the expression of NRDR was higher in testes of pig breeds with high androgen than that of pig breeds with low androgen (Jia *et al.*, 2023; Leung *et al.*, 2010). Research shows that single nucleotide polymorphism (SNP) of NRDR could be used as a genetic marker for pork quality (Hwang *et al.*, 2017).

NRDR had several isoforms in human and mouse, with different expression patterns and catalytic activities to substrates (Falqui *et al.*, 2023; Gan *et al.*, 2023; Huang *et al.*, 2023; Zhang *et al.*, 2009). In our previous study found that NRDR existed in the pig uterus (unpublished), but the expression and function of NRDR in uterus and the expression of different isoforms in the implantation stage of uterus have not been reported. Therefore, in this study, RT-qPCR, immunofluorescence and other methods were used to explore the expression of NRDR and its different isoforms in uterus of the model animal mouse. The results of this study will provide an experimental basis for the analysis of the molecular changes of uterus during uterine development and pregnancy.

MATERIALS AND METHODS

Experimental animals: The 7-week-old C57BL/6 mice purchased from Institute of Experimental Animal Resources, China Academy of Food and Drug Control were used in this experiment. The mice were raised in suitable environment. The animal experiments were conducted in accordance with the relevant provisions the Chinese Association for Laboratory Animal Sciences. Male and female mice were mated, and the detection of vaginal suppository was recorded as 0.5 days of pregnancy. Uterus, ovaries and serum were obtained from female mice at 0.5, 2.5, 4.5 and 6.5 days of normal pregnancy. Uteruses were obtained from female mice at 3D, 6D, 9D, 12D, 15D and 21D from birth to puberty. The tissues for every experiment were at least three samples and stored at -80℃.

Method

Real-time quantitative PCR (RT-qPCR): The process of RNA extraction was performed in an RNase-free environment. After sample collection, total RNA was extracted using RNAiso Plus (TaKaRa) according to the instructions. RNA concentration and purity were determined by Nano Drop 2000 (Thermo). The purified total RNA (1 μg) was used reversely transcribe cDNA according to Reverse Transcription Kit (TaKaRa).

In RT-qPCR TB GreenTM Premix Ex TaqTM Kit (TaKaRa) and Amplied Biosystems®7500 System (Amplied Biosystems) were used. This was performed in an. In the reaction, GAPDH was used as the internal reference gene, and $2^{-\triangle\triangle CT}$ was used for data analysis. The primers of NRDR-1, NRDR-2 and GAPDH for RT-qPCR were designed online in NCBI and synthesized by GENEWIZ. Primer sequences were shown in Table 1.

Immunofluorescence assay (IFA): Uterus from slaughtered mouse were immediately put in a fresh solution of 4% paraformaldehyde for 24 hours, then were embedded in OCT and quick-frozen with liquid nitrogen. The quick-frozen tissue was placed in OCT embedded adhesive, and the tissue was soaked at 4℃ for 15 min. Then the OCT embedded adhesive was taken to completely cover the quick-frozen tissue for 30 min and then uterine tissues were sliced into 10 μm sections. The specific experimental process of IFA was consistent with previous reports (Liu *et al.*, 2019). After hydration, frozen sections were repaired for antigen. Wash with PBS 3 times, 5 min each time; Block with 5% goat serum for 40 min. NRDR antibody (1:100, Abcam) was added and incubated at 4℃ overnight. Rabbit IgG incubating was as a negative control (NC). The samples were incubated with goat anti-Rabbit IgG (H+L), Alexa Fluor Plus 488 (1:500, Invitrogen) for 2 h at room temperature. Last 4',6-diamidino-2-phenylindole (DAPI) was incubated for 10 minutes on sections. Then the slides were observed using a microscope (Leica Micro-systems) and photographed.

Data sources for expression of NRDR in uterus of different model mice: The expression of NRDR in uterus of three model mice was analyzed, including: natural pregnancy decidualization (NPD), artificial decidualization (AD) and in vitro decidualization (IVD); according to the GEO database (GSE122376) (C. Wang *et al.*, 2020). In addition, the expression of NRDR in uterus during estrus and diestrus by using RNA-seq was analyzed and the data sourced from the GEO database (GSE131172) (Zhou *et al.*, 2022).

Statistical Analysis of Data: All data were analyzed by SPSS 25.0, and the experimental data were mean ± standard deviation (SD). The independent sample t-test was used for comparison between the two groups. A value of $p \leq 0.05$ was considered statistically significant.

RESULTS

NRDR expressed in mouse uterus: The expression of NRDR in mouse uterus was detected by IFA. NRDR was mainly expressed in the endometrium, uterine glands and uterine serosa of mouse (Fig. 1). This result indicated that NRDR might participate in regulating the decidualization process and cell proliferation of the endometrium in mouse.

The expression of NRDR in uterus gradually increased with the development of uterus: The expression levels of NRDR-1 and NRDR-2 which were

different isoforms for NRDR, were analyzed by RT-qPCR in uterus of mice at 3D, 6D, 9D, 12D, 15D and 21D from birth to puberty. The result showed that the expression of NRDR-1 (Fig. 2 A) and NRDR-2 (Fig. 2 B) in the uterus of mice gradually increased with the development of uterus, and the highest expression level was observed at 15 days of uterine development. There is a period of rapid growth as approach puberty in uterus. According to the above results, NRDR may be related to the development of uterus.

NRDR-2 expressed higher during estrus than diestrus in uterus: During estrus and diestrus, the cells of uterus are in different states, so there are obvious differences in their gene expression. Therefore, the expression of NRDR-1 and NRDR-2 was detected in mouse uterus during estrous and diestrus. NRDR was expressed in uterus of mice during estrus and diestrus. There was no difference in expression of NRDR-1 during estrous and diestrus of mouse uterus (Fig. 3 A). However, the expression of NRDR-2 in estrus was higher than that in diestrus (Fig. 3 B). These results showed that the expression of NRDR-2 in uterus of mice changed with the estrus cycle, suggesting that NRDR may be involved in the physiological activities of the estrus cycle in the form of NRDR-2.

NRDR was high expressed at 2.5 day of peri-implantation in uterus: To determine whether NRDR is associated with steroids regulated embryo implantation, the expression levels of NRDR were detected during early pregnancy in mouse uterus. RT-qPCR and radioimmunoassay were used to detect the levels of NRDR-1, NRDR-2, estradiol and progesterone in the uterus and serum during implantation. The expression of NRDR-1 and NRDR-2 in the peri-implantation uterus of mouse showed a trend of increasing first and then decreasing. In particular, the expression of NRDR-1 or NRDR-2 both reached peak at 2.5 day of peri-implantation and decreased at 6.5 days after implantation (Fig. 4C, D). Intrauterine estradiol levels remained low during implantation but showed a small peak at 4.5 days of gestation (Fig 4 B), whereas progesterone levels increased with the duration of gestation and stabilized in the second trimester (Fig 4 A). The expression of NRDR in the uterus during early pregnancy increased with the increase of progesterone; on the contrary, high levels of estradiol inhibited NRDR expression.

The expression of NRDR was obviously reduced in the abortion models of mouse: The expression of NRDR in uterus of three mouse decidualization models was analyzed using RNA-seq data, including: NPD, AD, IVD. The result showed the expression of NRDR was obviously reduced in abortion models (Fig 5A-C). In addition, the expression of NRDR in uterus during estrus and diestrus was analyzed by using RNA-seq and the result showed the expression of NRDR had no changed during estrus and diestrus (Fig 5 D).

Table 1 The primer sequence of RT-qPCR.

Genes	sequence(5'-3')
NRDR-1-UP	AGTTGGCCCCGAAGAACATT
NRDR-1-DOWN	CCCTGGTAGGCTGTTTCTGG
NRDR-2-UP	GGAGGTGTGGGACAAGGTTT
NRDR-2-DOWN	TGGAAGGAGCGACAGGTACT
GAPDH-UP	GCTGAGAACGGGAAGCTTGT
GAPDH-DOWN	GCCAGGGGTGCTAAGCAGTT

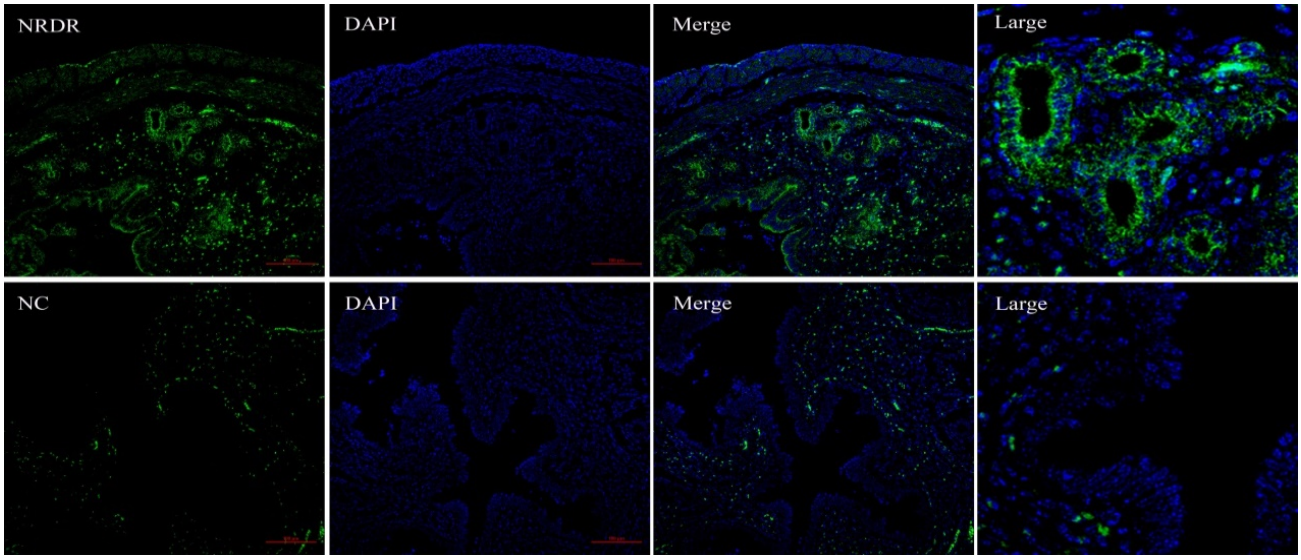

Fig.1 Expression of NRDR in mouse uterus by IFA

NRDR was labeled by green fluorescence， NC was negative control and all nuclei were labeled by blue fluorescence.

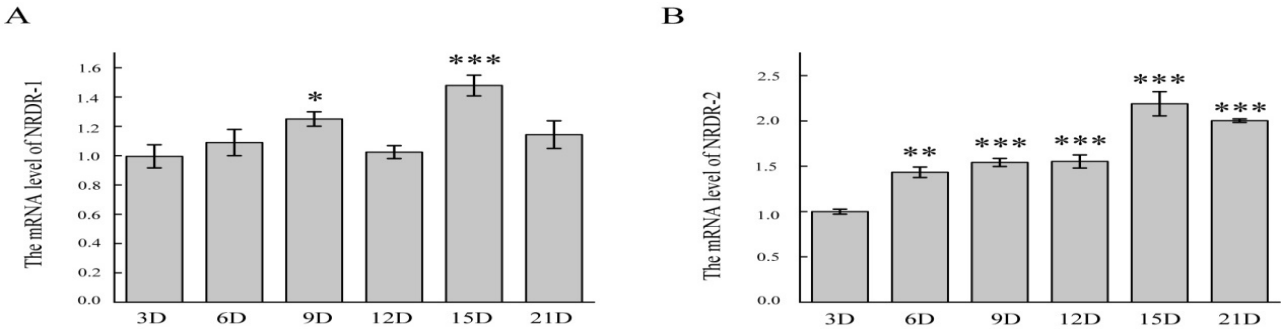

Fig.2 The expression of *NRDR* in the mouse uterus during development

NRDR-1(A) and NRDR-2(B) mRNA level was analyzed of in 3D, 6D, 9D, 12D, 15D and 21D of mouse uterus development by RT-qPCR. The experiments were normalized to NRDR mRNA level of 3D uterus. Data (N=3) were shown as means ± S.D. $^{*}P\leq0.05$.

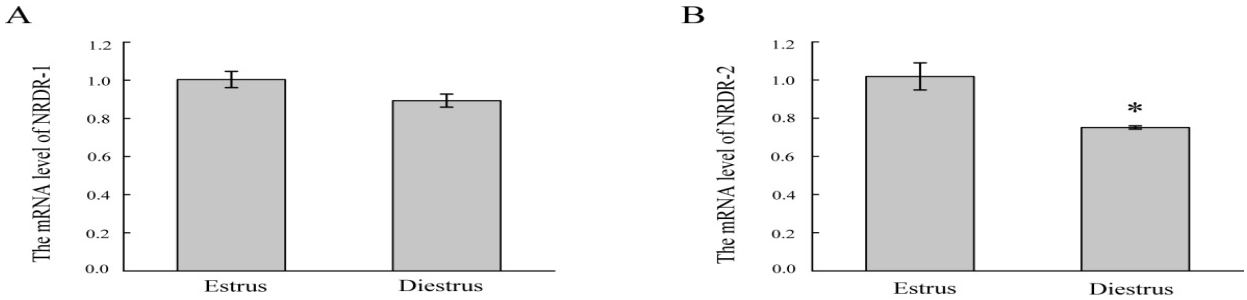

Fig. 3 The expression NRDR-1 and NRDR-2 in mouse uterus during estrous and diestruse

RT-qPCR was used to analyze NRDR-1(A) and NRDR-2(B) mRNA level in mouse uterus during estrus and diestrus. The experiments were normalized to NRDR mRNA level of estrous. Data (N=3) were shown as means ± S.D.$^{*}P\leqslant0.05$.

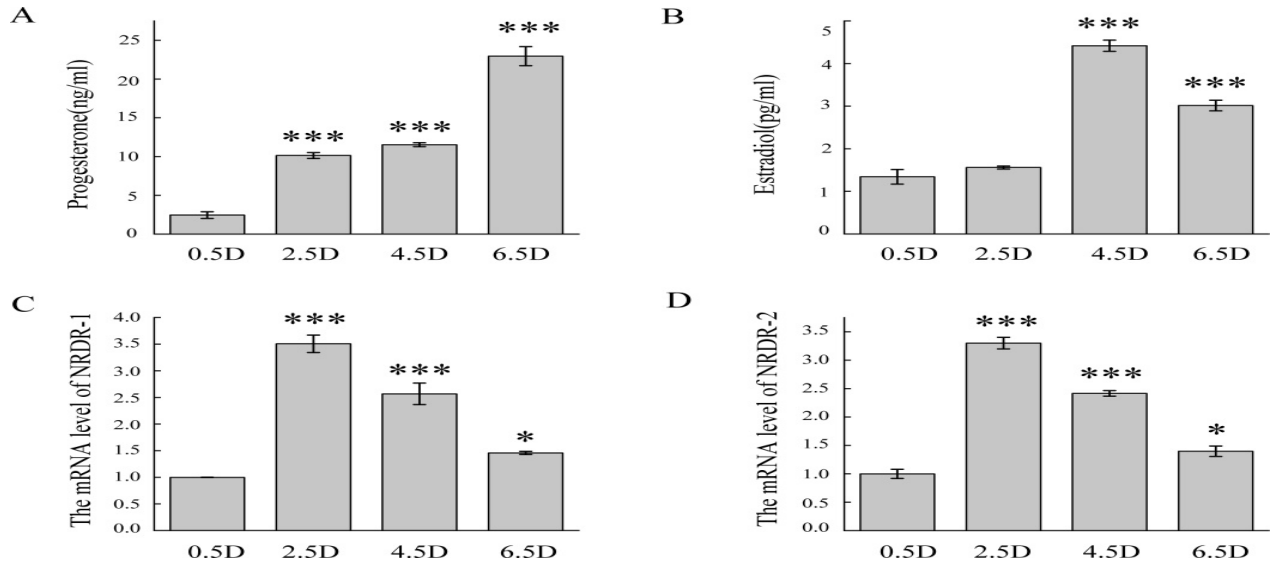

Fig. 4 The expression of NRDR and changes of estradiol and progesterone in mouse uterus during the different implantation period

(A) Progesterone and (B) Estradiol levels were measured by RIA respectively. Data were shown as means ± S.E.M. from 8 samples for each group. (C-D) RT-qPCR analyzed NRDR-1 (C) and NRDR-2 (D) mRNA level in 0.5D, 2.5D, 4.5D and 6.5D during implantation period of mouse uterus. The experiments were normalized to the data of 0.5D. Significant differences were indicated by * ($P \leq 0.05$) (ANOVA).

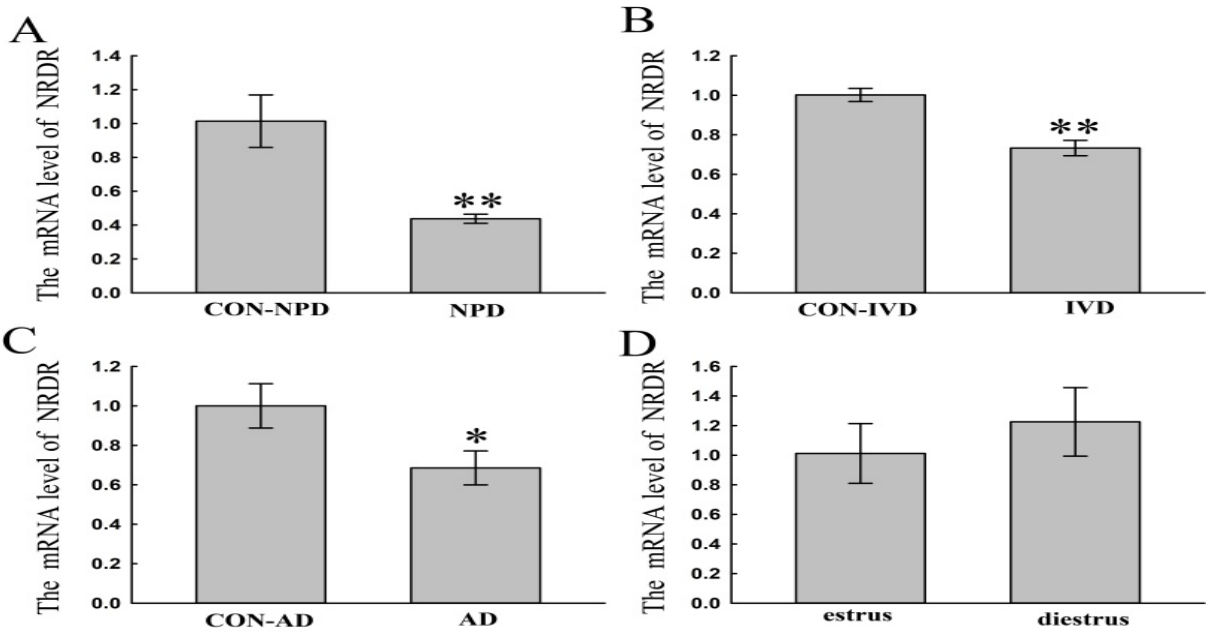

Fig. 5 the expression of NRDR in abortion model and estrous cycle in the mouse uterus

Data were analyzed of the expression of NRDR in abortion model and estrous cycle in the mouse uterus. The expression of NRDR was analyzed in natural pregnancy decidualization (NPD) mice and control mice (CON-NPD) (A), artificial decidualization (AD) mice and control mice (CON-AD) (B), in vitro decidualization (IVD) mice and control mice (CON-IVD) (C) and estrus and diestrus mice (D). The experiments were normalized to their respective control. Data (N=3) were shown as means ± S.D.*P≤0.05. **P≤0.01.

DISCUSSION

In mice, the process of uterine decidualization is very important for early pregnancy and embryo implantation. Estrogen and progesterone participate in regulating the process of uterine membrane degeneration (Cha *et al.*, 2014); however, the molecular mechanisms involved in the process of decidualization need to be further elucidated. At 0.5, 2.5 days of gestation, the luminal epithelium and the glandular epithelium of the mouse were rapidly proliferating under the regulation of estradiol in preparation for embryo implantation. From day 2.5 days of gestation, the uterine lumen epithelium began to differentiate as progesterone levels increased (Tamada *et al.*, 1990). At 4.5 days of gestation in mice, the blastocyst will enter the endometrium (Tabibzadeh *et al.*, 1995). Successful implantation of the embryo requires both the invasion of blastocyst trophoblast cells and the acceptance of the endometrium so that they can interact in the future. Day 4.5 and day 6.5 were important time points for embryo implantation and trophoblast invasion (Hancock *et al.*, 2023; *et al.*, 1995; Yuan *et al.*, 2023). In this study, the expression of NRDR-1 or NRDR-2 both reached peak at 2.5 day of peri-implantation and decreased at 6.5 days after implantation. According to the expression of NRDR, we speculate that NRDR plays an important role in the differentiation of uterine epithelium in early pregnancy.

In mice, the development of uterine glands begins with 5D (5 day after birth) and completes with 15D and endometrial glands is crucial for embryo implantation (Cooke *et al.*, 2013; Jefferson *et al.*, 2020). About 5D, the glandular epithelium gradually formed (Cooke *et al.*, 2012; Kurita *et al.*, 2001). From 7D to 10D, epithelial invaginations (buds) grew and invaded to the stroma (Branham *et al.*, 1985; Brody *et al.*, 1989). Until 15D, the mouse uterus developed completely (Hondo *et al.*, 2007; Hu *et al.*, 2004; Vue *et al.*, 2020). In this study, the expression of NRDR was increased on6D and peaked on 15D. According to the above, NRDR was related to the development of glandular epithelium during mouse uterine development. The above results were consistent with the results of immunofluorescence which NRDR was highly expressed in mouse uterine glands.

Previous studies have found that several genes play a regulatory role in the formation and implantation of uterine glands, such as Wnt7a, Sox17, Wnt4, Foxa2 and HOXA10 (Cooke *et al.*, 2013; Guimaraes-Young *et al.*, 2016; Jefferson *et al.*, 2020). This study found that NRDR was mainly expressed in the endometrium, uterine glands and uterine serosa of mice in utero, and its expression pattern was similar to the expression of Foxa2 and HOXA10 genes in uterus (Jefferson *et al.*, 2020). According to the above, we deduced that NRDR is also involved in endometrial development and affects embryo attachment and decidualization.

The function of NRDR in reproduction has been partially reported. NRDR was associated with testosterone levels in boars (Grindflek *et al.*, 2010; Moe *et al.*, 2009; Moe *et al.*, 2007). In addition, our previous study found that NRDR could inhibit estradiol synthesis in porcine ovarian granulosa cells (Liu *et al.*, 2019). In this study, different methods were used to demonstrate that NRDR is expressed in the mouse uterus, and its expression is changed in different physiological processes. In conclusion, NRDR plays an important role in mammalian reproductive system.

Previous research found that NRDR had two isoforms in human and mouse, and different isoforms had different expression patterns in tissues (Falqui *et al.*, 2023; Gan *et al.*, 2023; Huang *et al.*, 2023; Zhang *et al.*, 2009). In this study, we revealed the expression of NRDR isoforms in different physiological states of uterus, and found that the two reported NRDR isoforms were both expressed in the uterus of mice. The expression of NRDR-2 changed significantly during the estrus cycle. This experimental result is similar to previous reports, and further enriched the expression distribution of different isoforms of NRDR in tissues.

On the other hand, the expression of NRDR in uterus of was significantly down-regulated in decidualization models of mouse (Wang *et al.*, 2020). Combined with the results of this study, the expression of NRDR changed during the implantation of mouse embryos suggest that NRDR might play an important role in the physiological changes of uterus during the implantation of mouse embryos.

Conclusion: This study found that NRDR was mainly expressed in the endometrium of mice. The expression of NRDR-1 and NRDR-2, two different isoforms of NRDR, showed a gradual upward trend during the development of mouse uterus. Meanwhile, the expression of NRDR-2 in estrus was significantly higher than that in diestrus. The expression of NRDR increased during the process of embryo recognition and implantation in the peri-implantation uterus of mice, but decreased after implantation. These results suggest that NRDR might play an important role in uterus and provide a basis for further study of the function of NRDR in uterus.

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Authors' Contributions: Jiankai SHI, Xiaoying HE, Ying LIU designed experiments; Ying LIU and Jiankai SHI analyzed data. Jiankai SHI, Shuxin LI, Yang LIU, Lei TIAN and Shujun LIU collected samples, Jiankai SHI, Jiwei LIU and Shuxing LI performed experiments; Ying LIU, Jiankai SHI and Libing MA wrote the manuscript. All authors agreed to publish this article.

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